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Hydroxyl radical production and autoxidative glycosylation

Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing

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Protein exposed to glucose is cleaved, undergoes conformational change and develops fluorescent adducts ('glycofluorophores'). These changes are presumed to result from the covalent attachment of glucose to amino groups. We have demonstrated, however, that the fragmentation and conformational changes observed are dependent upon hydroxyl radicals produced by glucose autoxidation, or some closely related process, and that antioxidants dissociate structural damage caused by the exposure of glucose to protein from the incorporation of monosaccharide into protein. We have also provided further evidence that glycofluorophore formation is dependent upon metal-catalysed oxidative processes associated with ketoaldehyde formation. If experimental glycation is an adequate model of tissue damage occurring in diabetes mellitus, then these studies indicate a therapeutic role for antioxidants.

INTRODUCTION

Protein glycation as a model of tissue damage associated with diabetes mellitus and ageing

The exposure of macromolecules *in vitro* to concentrations of glucose representative of hyperglycaemia is widely considered a relevant model for the functional degeneration occurring (particularly to low-turnover and extracellular structures) in diabetes mellitus and ageing (Cerami, 1986). Protein, for example, undergoes structural alterations and develops novel fluorophores (Pongor *et al.*, 1984) similar to those found to correlate with pathological tissue alterations in diabetic individuals (Monnier *et al.*, 1986).

Glucose autoxidation

It appears to be assumed that the covalent attachment of glucose to amino groups via the Amadori pathway, and subsequent rearrangement and dehydration reactions, during such glycation studies, are sufficient to account for the structural changes observed (Cerami, 1986; Pongor *et al.*, 1984; Harding, 1985). Glucose, however, in common with other α-hydroxyaldehydes (Wolff *et al.*, 1984), is prone to transition metal-catalysed oxidation (via its enediol) generating H_2O_2, reactive intermediates, such as the hydroxyl radical, as well as ketoaldehydes (Wolff & Dean, 1987*a*). Oxidative chemistry of glucose could well contribute to macromolecular alterations associated with experimental glycation.

Autoxidation-derived ketoaldehydes and protein glycation

We have shown previously, consistent with this possibility, that the ketoaldehyde products of glucose autoxidation contribute substantially to total monosaccharide covalently attaching to protein during the exposure of protein to glycose *in vitro* (Wolff & Dean, 1987*a*,*b*). Glucose autoxidation is slow, but the amounts of ketoaldehyde formed over the typical time courses of glycation studies are in the range consistent with some contribution to total protein-attached monosaccharide and also contribute to chromo- and fluoro-phoric alterations (Wolff & Dean, 1987*a*,*b*; 1988).

Oxidative stress in diabetes and ageing and the role of transition metal and autoxidizable substances

There is considerable evidence suggesting that oxidative stress plays a role in tissue damage associated with diabetes (Wolff, 1987) and ageing (Harman, 1981). The source of this inappropriate oxidation is not known, but could be related to an increase in the concentration of redox catalysts, and/or substances prone to peroxide and free-radical generation, such as monosaccharides. For example, Cu^{2+}, which readily catalyses reactions involving H_2O_2 and free radicals, increases in concentration in plasma with age (Harman, 1965) and in diabetes (Noto *et al.*, 1983). The ability of such biological Cu^{2+} to catalyse radical reactions is not well defined (Gutteridge *et al.*, 1985), but Cu^{2+} levels are also known to increase with age in the normal lens, and more so in idiopathic cataract associated with ageing (Nath *et al.*, 1969), or with other risk factors, such as ocular inflammation (McGahan & Bito, 1983). This suggests a role for transition metal-catalysed enediol oxidation in tissue damage associated with age and diabetes (Wolff *et al.*, 1987) and led us to consider, here, the role of free radicals and H_2O_2, produced by glucose enediol autoxidation, in the structural alterations induced by the exposure of protein to glucose.

METHODS

Measurement of protein structural change

Bovine serum albumin (Boehringer; Fraction V), was ^{14}C-radiomethylated and subsequently assessed for fragmentation by glucose as described previously (Hunt

Abbreviations used: BSA, bovine serum albumin; PAGE, polyacrylamide-gel electrophoresis; DETAPAC, diethylenetriaminepenta-acetic acid.

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et al., 1988). SDS/polyacrylamide-gel electrophoresis (PAGE) of albumin was also performed as described previously (Hunt *et al.*, 1988). Tryptophan fluorescence alterations were monitored at 350 nm [280 nm excitation (Wolff & Dean, 1987*a*)].

Hydroxyl radical detection

The hydroxylation of benzoate by monosaccharides was measured by fluorescence [308 nm excitation (EX); 410 nm emission (EM)], using a salicylate standard (Gutteridge, 1987) and appropriate controls. The formation of malonaldehyde (expressed as absorbance at 532 nm), formed as a result of deoxyribose oxidation (Halliwell & Gutteridge, 1981), was estimated by incubation of a 5% trichloroacetic acid-treated reaction aliquot with an equal volume of 50 mM-thiobarbituric acid for 30 min at 100 °C. Glucose generated comparably little thiobarbituric acid-reactive material in the absence of deoxyribose.

Ketoaldehyde and glycofluorophore formation

'Glycofluorophores' (novel fluorophores formed as result of the exposure of protein to glucose) were measured using excitation at 350 nm and emission at 415 nm (Wolff & Dean, 1987*a*). Ketoaldehydes were measured using the Girard T reagent as described previously (Wolff & Dean, 1987*a*).

Assessment of glucose incorporation into protein

The incorporation of label from D-[U-^{14}C]glucose into protein and confirmation of the absence of protein-reactive impurities in commercial radioactively labelled glucose (Trueb *et al.*, 1980), obtained from Amersham, were performed as described previously (Wolff & Dean, 1987*a*).

Details of the experimental regimes can be found in the legends to Figures. All results are representative of at least two experiments and are presented, where appropriate, as the mean ± S.D. for triplicate assays.

RESULTS

Glucose-induced protein fragmentation and benzoate hydroxylation

The glucose-induced fragmentation of ^{14}C-radiomethylated bovine serum albumin [assessed by the release of radiolabel into a trichloracetic acid-soluble fraction (Hunt *et al.*, 1988)], and the corresponding hydroxylation of benzoate (Gutteridge, 1987) during exposure to glucose under various conditions, are illustrated in Fig. 1. The addition of Cu^{2+} ions, which form oxidizing/hydroxylating agents in the presence of H_2O_2 (Chan & Kesner, 1980), stimulated these processes (by 1.8 and 4.6-fold respectively), whereas diethylene-triaminepenta-acetic acid (DETAPAC), which sequesters the trace amounts of transition metal necessary for glucose enediol oxidation (Wolff & Dean, 1987*a*,*b*), inhibited them (by 90.4% and 96.8% respectively). The polyhydric alcohol sorbitol, considered a scavenger selective for hydroxyl radicals at physiological pH [since no other radicals appear to be capable of abstracting hydrogen from aliphatic C–H bonds under these conditions (Koppenol & Butler, 1985)], similarly inhibited benzoate hydroxylation and protein cleavage (96% and 75.3% respectively).

Confirmation of fragmentation by SDS/PAGE and the effect of other monosaccharides

SDS/PAGE analysis of albumin exposed to glucose under similar conditions (Fig. 1, inset) confirmed that

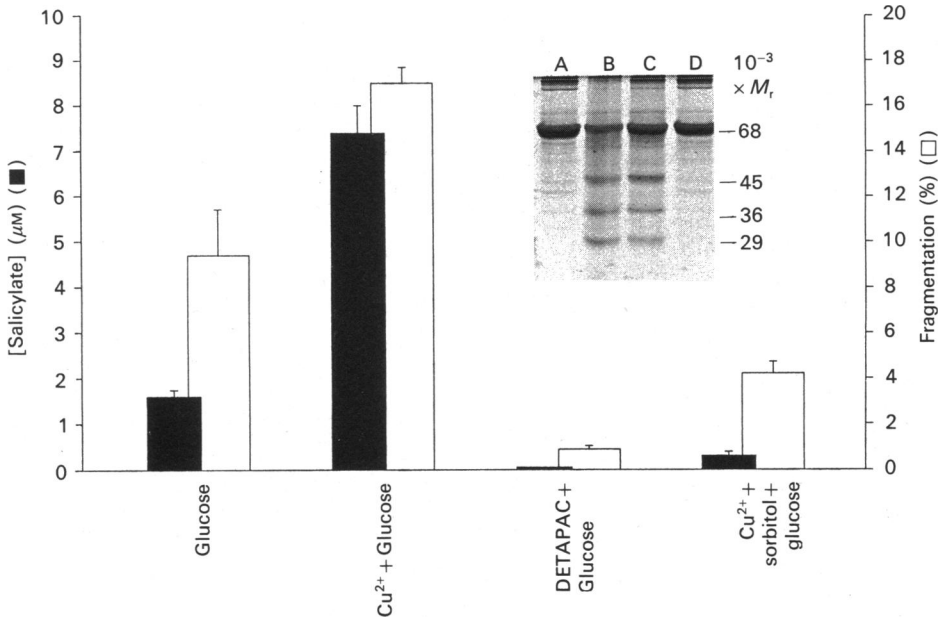


Fig. 1. Glucose induces protein fragmentation which correlates with its ability to hydroxylate benzoate under various conditions

^{14}C-radiomethylated BSA (1 mg/ml) or benzoate (1 mM) was incubated with 25 mM-glucose alone, or in the presence of 100 μM-Cu^{2+}, 1 mM-DETAPAC or 250 mM-sorbitol together with 100 μM-Cu^{2+} in 100 mM-potassium phosphate buffer, pH 7.4, for 8 days at 37 °C. SDS/PAGE of albumin (1 mg/ml) exposed to glucose under analogous conditions was performed as described previously (Hunt *et al.*, 1988). Track A, control-incubated albumin; B, Cu^{2+} (100 μM), glucose (25 mM), albumin; C, glucose, albumin; D, glucose, DETAPAC (1 mM), albumin.

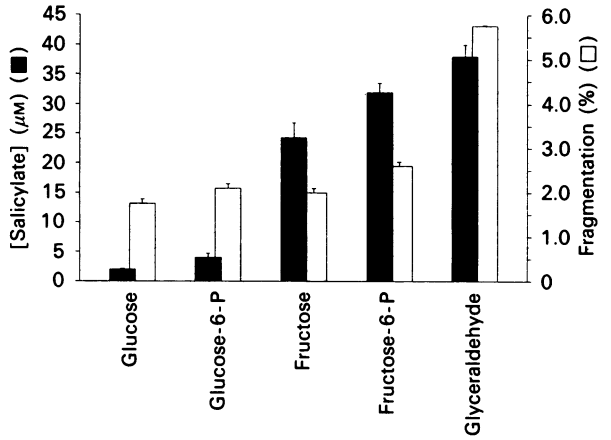


Fig. 2. Various monosaccharides hydroxylate benzoate and stimulate protein fragmentation

Monosaccharide (5 mM) was incubated over 4 days at 37 °C in 100 mM-potassium phosphate buffer, pH 7.4, 1 mM-benzoic acid (or 1 mg of ^{14}C-radiomethylated BSA/ml) and 100 μM-Cu^{2+}. Protein fragmentation was measured as in Fig. 1, in the presence of 100 μM-Cu^{2+} over 4 days. Abbreviation: -6-P, 6-phosphate.

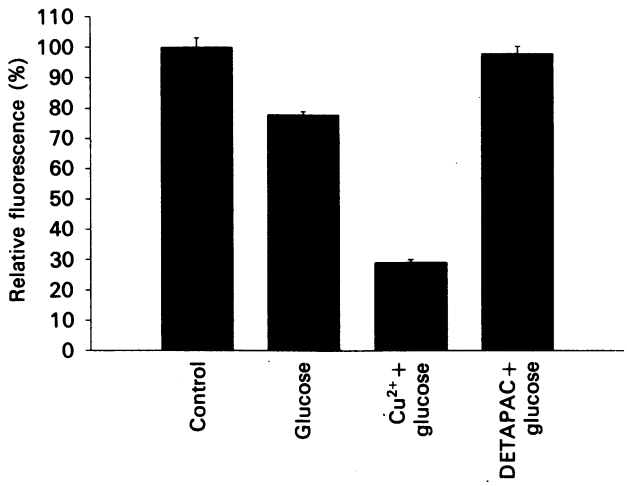


Fig. 3. Transition metal dependence of glucose-induced tryptophan fluorescence quenching

BSA (1 mg/ml) was incubated with 25 mM-glucose in the presence of 10 μM-Cu^{2+} or 1 mM-DETAPAC over 4 days under the conditions described in Figs. 1 and 2.

true fragmentation occurred and produced a limited fragmentation pattern strongly reminiscent of the normoxic exposure of albumin to hydroxyl radicals generated by the gamma-radiolysis of water (Dean *et al.*, 1986), or peroxide in the presence of Cu^{2+} (Hunt *et al.*, 1988), in contrast to the more generalized dissimilation produced by hypochlorite (Dean *et al.*, 1986), which generates protein-bound proximal oxidants. This suggests that the fragmenting agents are mainly produced in free solution and possess a specificity and mechanism of reaction akin to the hydroxyl radical (Wolff *et al.*, 1986). Fragmentation of protein was produced by glucose in the presence or absence of added Cu^{2+} (Fig. 1, inset, tracks B and C respectively), but was inhibited by DETAPAC in both cases (track D). Other monosaccharides stimulated the protein fragmentation to an extent broadly reflecting their ability to hydroxylate benzoate (Fig. 2) and, similarly, other polyaminocarboxylates, such as EDTA inhibited fragmentation in the presence and absence of added transition metal (results not shown).

The role of transition metal in fragmentation

The enhancement of the fragmentation effect caused by the addition of Cu^{2+}, presumably reflects reaction of the transition metal with the low levels of accumulating H_2O_2 (Wolff & Dean, 1987*a*). It will not increase the rate of glucose autoxidation [at least as judged by ketoaldehyde accumulation (Wolff & Dean, 1987*a*; see below)] since physiological buffers provide concentrations of adventitious catalytic transition metal in excess of the low amounts of autoxidation-susceptible enediol present (Finkelstein *et al.*, 1980; Wolff & Dean, 1987; Wolff *et al.*, 1984). Addition of further transition metal cannot stimulate an already transition metal-saturated system (Wolff & Dean, 1988). We did note, however, that increasing the concentration of bovine serum albumin (BSA) in these experiments lessened the fragmentation of BSA stimulated by Cu^{2+}, consistent with the ability of BSA to bind Cu^{2+} and act as an inhibitor of reactions involving H_2O_2 and this particular transition metal (Halliwell, 1988).

Transition metal involvement in glucose-induced conformational change

Glucose-induced tryptophan fluorescence quenching (275 nm excitation; 350 nm emission) associated with BSA conformational change and consequent alterations in the tryptophan microenvironment (Shaklai *et al.*, 1984; Dean *et al.*, 1986; Wolff & Dean, 1987*a*), was also subject to manipulation by modifiers of free-radical reactions (Fig. 3). Cu^{2+} produced a 3.2-fold enhancement of glucose-induced quenching, whereas it was inhibited 91% by DETAPAC (Fig. 3). Conformational change, assessed either by fluorescence quenching or by tryptic susceptibility, appears to be a precursor to protein fragmentation induced by hydroxyl radicals (Hunt *et al.*, 1988; Wolff & Dean, 1986).

Hydroxyl radicals and protein fragmentation

The role of oxidizing agents in protein structural change induced by glucose was further investigated in competition experiments using benzoic acid and deoxyribose [which reacts with the hydroxyl radical to form malonaldehyde (Halliwell & Gutteridge, 1981)] as hydroxyl-radical scavenger/detectors (Fig. 4). Both agents inhibited protein fragmentation (by 53.5% and 35.3% respectively, at 1 mM (Fig. 4), and this protection was associated with simultaneous hydroxylation of the benzoate and oxidation of deoxyribose to malonaldehyde (Fig. 4, insets *a* and *b*). Given that the hydroxyl-radical scavenger sorbitol was a potent inhibitor of glucose-induced protein fragmentation (Fig. 1), it can be concluded that the hydroxyl radical [or a similarly reactive hydroxylating/oxidizing species (Rush & Koppenol, 1987)] is responsible for a substantial part of the structural damage experienced by protein exposed to glucose *in vitro*.

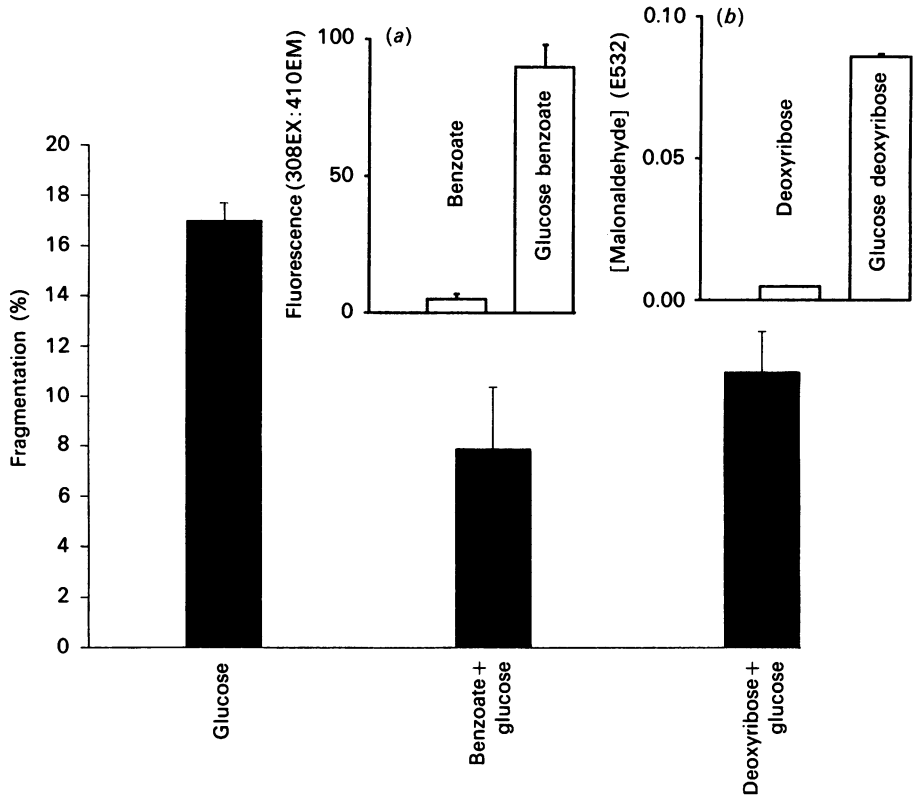


Fig. 4. Hydroxyl radical scavenger/detectors protect against glucose-induced fragmentation and reveal hydroxyl radical formation

Fragmentation by 25 mM-glucose in the presence of 100 μM-Cu^{2+} was assessed using radiomethylated albumin (1 mg/ml). Benzoate and deoxyribose were added at a concentration of 1 mM. Fluorescence of benzoate hydroxylation products (inset *a*) was assessed after precipitation of protein with trichloroacetic acid and retitration to pH 7.2 using concentrated potassium phosphate buffer. Malonaldehyde (inset *b*), formed from deoxyribose in the presence of glucose, was measured as described in the Methods section.

Hydroxyl rather than superoxide radical damage in glycation-associated protein damage

The rate of production of the protein-damaging oxidant by glucose is too slow for visualization by the e.s.r. technique of spin-trapping [at least using dimethylpyrroline-*N*-oxide (S. P. Wolff, unpublished observation)], but rapid enough to enter, cumulatively, the order-of-magnitude concentration range of the protein over the time course of the experiment (as judged by the concentration of fluorescent benzoate hydroxylation products), and is thus consistent with the extent of protein fragmentation observed, given that oxidative protein fragmentation by hydroxyl radicals (in the presence of oxygen) is a very efficient process (Wolff & Dean, 1986). The contribution of superoxide radical ($O_2^{-\cdot}$) production to protein structural change induced by exposure to glucose can, in contrast, be ignored since $O_2^{-\cdot}$, and its conjugate acid, the hydroperoxyl radical ($HO_2^{\cdot}$) react poorly with protein (Wolff & Dean, 1986; Dean *et al.*, 1986).

The role of H_2O_2 and 'site-specific radical attack'

Although the steady-state concentrations of H_2O_2 were found to lie at the detection limits (2.5 μM) of the sensitive Fe^{2+}/Xylenol Orange assay (Gupta, 1973) or the horseradish-peroxidase-catalysed oxidation of 4-aminoantipyrine/phenol (Frew *et al.*, 1983), the role of H_2O_2 in the generation of the fragmenting agent could be demonstrated by the inhibitory effect of catalase (Fig. 5). Increasing concentrations of catalase produced increasing extents of inhibition of fragmentation, which approached a maximum of *ca.* 75% (72.2% at 1100 units/ml, relative to a heat-inactivated control), comparable with the 75.3% inhibition of fragmentation shown by sorbitol, at a 2×10^5-fold excess (Fig. 1). This absence of total inhibition may be explained in the context of site-specific radical attack (Czapski, 1978) in which antioxidants cannot interrupt the local attack of oxidants at the site in which they are generated; for example, in the case of hydroxyl radicals produced by the reaction of H_2O_2 with transition metal at protein surfaces (Gutteridge & Wilkins, 1983). A maximal inhibition of considerably less than 100% for a radical-mediated biological reaction is thus not unexpected.

Antioxidants dissociate glycation from protein damage by glucose

In contrast, the possibility that protein fragmentation occurs by peptide hydrolysis, or some other non-oxidative autolytic mechanism, subsequent to the covalent attachment of monosaccharide to amino groups, is not consistent with the observation that sorbitol, deoxyribose and benzoate significantly inhibited fragmentation (Figs. 1 and 4), whilst having an insignificant effect on the incorporation of monosaccharide into protein {measured by the incorporation of radioactivity from [U-^{14}C]-

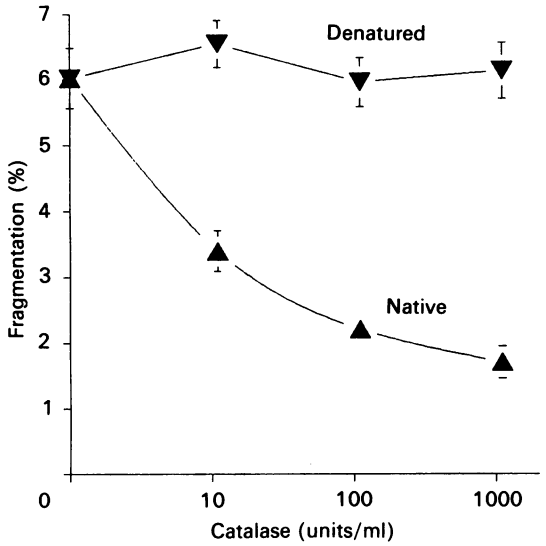


Fig. 5. Catalase protects against glucose-induced protein degradation

Radiomethylated albumin (1 mg/ml) was incubated with 25 mM-glucose and 100 μM-Cu^{2+} in the presence of catalase (Sigma; 11000 i.u./mg) over 3 days. Denatured catalase was prepared by incubation of the protein at 100 °C for 15 min.

glucose as described previously (Wolff & Dean, 1987*a*,*b*)} in the presence or absence of added Cu^{2+} (Table 1). Structural change induced by the exposure of protein to glucose can thus be dissociated from the incorporation of monosaccharide into protein *per se*. This is consistent with previous studies in which it was shown that sodium cyanoborohydride (which selectively reduces aldimine Schiff bases and probably acts as an antioxidant) accelerates protein glycation, but protects against protein confirmational change (Wolff & Dean, 1987*a*).

The role of transition metal in monosaccharide attachment to protein

The lower extent of monosaccharide incorporation into protein in the presence of added Cu^{2+} (Table 1) can be largely accounted for by the associated fragmentation of protein and the loss of label into the trichloroacetic acid-soluble fraction, at the concentration of protein employed. In the absence of added transition metal, DETAPAC produced a 29% inhibition of monosaccharide attachment, which is similar to that observed previously (Wolff & Dean, 1987*a*,*b*). This is supportive of the partial role of autoxidation-derived ketoaldehydes in glycation. In the presence of added Cu^{2+}, DETAPAC restores incorporation to the level between that observed without added Cu^{2+} in the presence and absence of DETAPAC. Since Cu^{2+}–DETAPAC complexes retain some ability to catalyse the oxidation of glucose (Wolff & Dean, 1987*a*) in a dose-dependent fashion, restoration to the level produced by DETAPAC in the absence of Cu^{2+} is not possible. An additional factor in the lower extent of monosaccharide incorporation in the presence of Cu^{2+} may be the ability of the transition metal to cause a slight lowering of the steady-state concentration of ketoaldehyde in solutions

Table 1. Incorporation of monosaccharide into protein

The incorporation of label from D-[U-^{14}C]glucose into protein (1 mg/ml), after 8 days of incubation in 100 mM-potassium phosphate buffer at 37 °C was measured as described in the Methods section. The concentrations of agents used to attempt to modify the incorporation of monosaccharide into protein were 1 mM for deoxyribose, benzoate and DETAPAC and 100 μM for Cu^{2+}. The final concentration of glucose was 25 mM, containing 2.5 μCi of D-[U-^{14}C]glucose/ml.

	Incorporation of monosaccharide into protein (mol of monosaccharide/mol of protein)	
Condition	With Cu^{2+}	Without Cu^{2+}
Control	2.50±0.25	6.24±0.33
Sorbitol	2.30±0.08	5.95±0.45
Benzoate	2.66±0.07	5.75±0.66
Deoxyribose	2.50±0.08	5.82±0.39
DETAPAC	5.40±0.32	4.43±0.30

of autoxidizing glucose (see below; Fig. 6). Neither sorbitol, benzoate nor deoxyribose influenced the generation of ketoaldehydes by glucose autoxidation, however, as measured by the Girard T reagent [(Mitchel & Birnboim, 1977; Wolff & Dean, 1987*a*,*b*); results not shown].

Monosaccharide oxidation, glycofluorophore development and the role of transition metal

The effect of agents modifying protein fragmentation by glucose upon the development of novel fluorophores (Fig. 6) was particularly revealing and strengthened the hypothesis that autoxidation-derived ketoaldehydes contribute to 'glycofluorophore' development (Wolff & Dean, 1987*a*,*b*). DETAPAC inhibited ketoaldehyde formation and glycofluorophore production to an extent observed previously (Wolff & Dean, 1987*a*,*b*), confirming that autoxidation-derived ketoaldehydes play a role of glycofluorophore development. Less ketoaldehyde was detectable in solutions of autoxidizing glucose containing protein (Fig. 6, inset), supportive of the view that ketoaldehydes formed from glucose autoxidation react with protein (Wolff & Dean, 1987*a*,*b*). DETAPAC also decreased ketoaldehyde formation, and this inhibition was greater if protein was absent [83% versus 74% inhibition in the presence of protein (Fig. 6, inset)], consistent with the previous study (Wolff & Dean, 1987*a*) and the hypothesis that Cu^{2+} bound to high-affinity sites on BSA cannot be removed by chelating agents and may continue to catalyse enediol oxidation (Marx & Chevion, 1986; Wolff & Dean, 1987*a*). Cu^{2+} also decreased glycofluorophore development and simultaneously decreased ketoaldehyde formation (Fig. 6). Although the addition of transition metal does not stimulate glucose autoxidation *per se* [for reasons discussed above (Wolff & Dean, 1987*a*,*b*)], it appears that excess transition metal can catalyse the degradation of α-ketoaldehydes and thus lower their steady-state concentration.

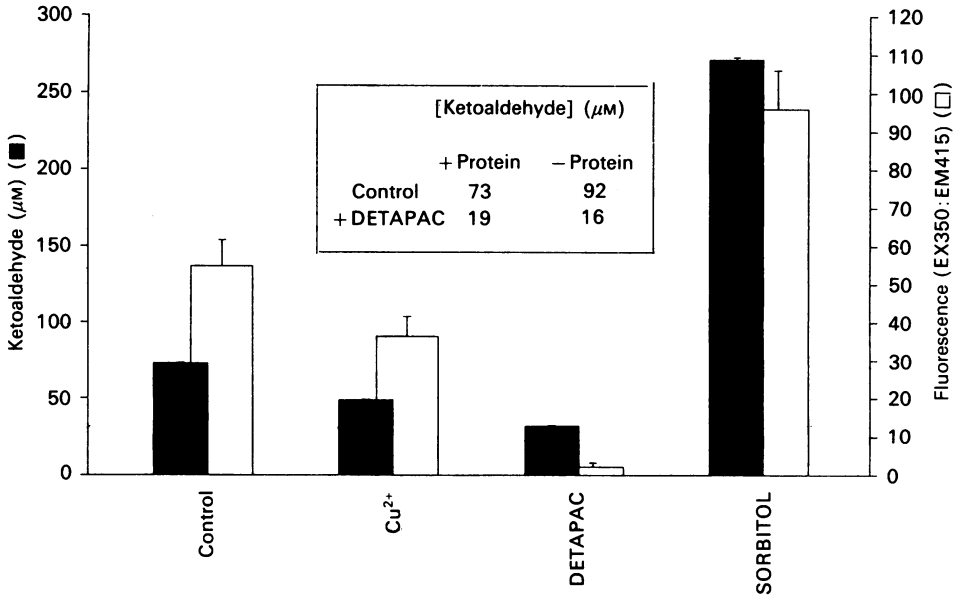


Fig. 6. Glycofluorophore development and ketoaldehyde formation

BSA (1 mg/ml) was incubated with 25 mM-glucose alone, with 100 μM-Cu^{2+}, 1 mM-DETAPAC or 250 mM-sorbitol for 4 days at 37 °C in 100 mM-potassium phosphate buffer, pH 7.4. The inset shows the extent of ketoaldehyde formation (measured by the Girard T reagent) in the presence and absence of protein (1 mg of BSA/ml) or DETAPAC (1 mM) under identical conditions.

Sorbitol oxidation and dissociation of protein damage from glycofluorophore development

Sorbitol, in contrast, substantially increased ketoaldehyde and glycofluorophore formation (Fig. 6). This was a surprising result and we speculated that sorbitol, when reacting with hydroxyl radicals produced by glucose autoxidation, could generate open-chain hydroxyaldehydes [subsequent to $HO_2^{\cdot}$ elimination from α-hydroxyalkylperoxyl radicals (Bothe *et al.*, 1978)], which were subject to facile autoxidation, generating further ketoaldehydes. We thus exposed sorbitol to hydroxyl radicals (generated by the gamma-radiolysis of water) and incubated it under analogous conditions to those used for the study described in Fig. 6. Table 2 shows that sorbitol subjected to attack by hydroxyl radicals is indeed prone to ketoaldehyde formation, compared with non-irradiated sorbitol. The ketoaldehydes generated by the autoxidation of sorbitol oxidation products contribute to glycofluorophore development. The result with sorbitol also indicates that glycofluorophore formation cannot be simply equated with structural damage when protein is exposed to high levels of monosaccharide, but is dependent upon the chemistry of other components in the system (in particular, upon their ability to form aldehydes on reaction with hydroxyl radicals), since sorbitol inhibits protein fragmentation (Fig. 1), but stimulates glycofluorophore development (Fig. 6). This observation may be of relevance to studies of tissue fluorescence in human diabetes (Monnier *et al.*, 1986), although we do not wish to suggest that sorbitol acts as an antioxidant *in vivo*.

Table 2. Generation of ketoaldehydes from hydroxyl radical-oxidized sorbitol

Sorbitol (100 mM) was exposed (in deionized water) to 100 nmol of hydroxyl radical/ml generated by the gamma-radiolysis of water in the 2000 Ci ^{60}Co source at Brunel University [described previously (Wolff & Dean, 1986)], diluted with an equal volume of 100 mM-potassium phosphate buffer, pH 7.4, and incubated at 37 °C for 3 days. The content of ketoaldehydes in the irradiated and incubated sample was then assessed by using the Girard T reagent and non-irradiated or non-incubated samples as comparisons.

	[Ketoaldehyde] (μM)	
	Pre-incubation	Post-incubation
Control sorbitol	0.0	0.0
Irradiated sorbitol	1.2	26.3

DISCUSSION

Possible mechanisms of hydroxyl radical production

Protein fragmentation and conformational changes induced by the exposure of protein to glucose are dependent upon hydroxylating/oxidizing agents generated in the presence of transition metal from H_2O_2 produced by glucose autoxidation, or some closely related process. There are two routes of monosaccharide attachment to protein at physiological levels of glucose; that which occurs via autoxidative glycosylation and that proceeding via the conventional Amadori rearrangement (Wolff & Dean, 1987*a*,*b*, 1988). Amadori product autoxidation [which is a process occurring at physiological pH (Ahmed *et al.*, 1986)] could, therefore, contribute to this hydroxyl radical-mediated damage. However, DETAPAC, which inhibits both glucose and Amadori product autoxidation (Ahmed *et al.*, 1986;

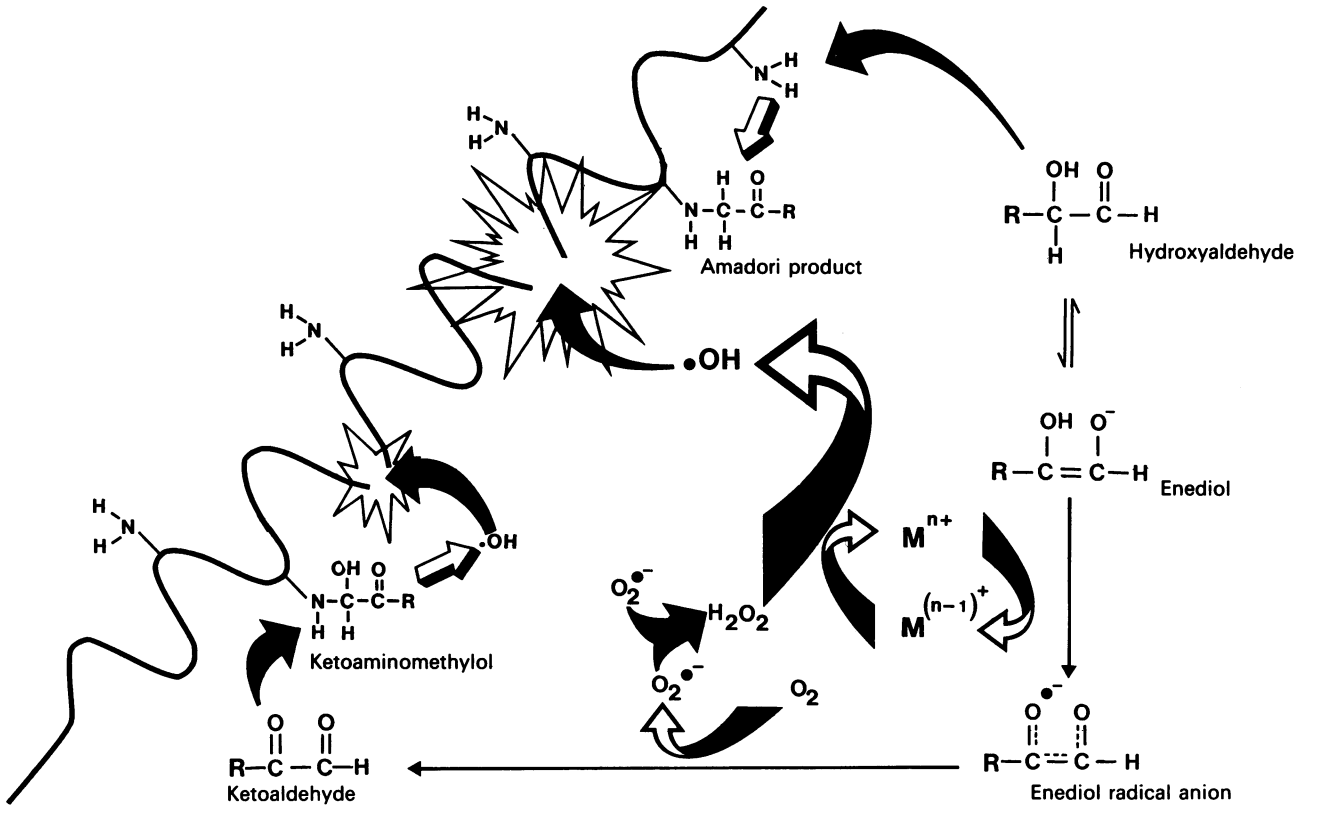
Wolff & Dean, 1988), decreases monosaccharide incorporation from glucose into protein, rather than increasing it, in conflict with predictions from studies of the autoxidation of the model Amadori adduct, *N*-formyl-*N*-fructolysine (Ahmed *et al.*, 1986; Wolff & Dean, 1988).

This suggests that Amadori product autoxidation is probably not an important process, at least in determining the overall extent of glycation adducts *in vitro*. Furthermore, even although there is some evidence to suggest that the rate of Amadori product autoxidation may be an order of magnitude greater than that of glucose or fructose, under analogous conditions [by comparison with the data in Wolff & Dean (1987*a*), Fig. 2 in this paper and Ahmed *et al.* (1986)], the total concentration of Amadori product is no more than 1/250th of the total free glucose under these conditions. Hydroxyl radical production by Amadori product autoxidation would, however, be 'site-specific' and could thus contribute to that component which is refractory to inhibition by hydroxyl radical scavengers *in vitro*, as well as being of significance *in vivo* (Ahmed *et al.*, 1986). Ketoaldehyde–protein adducts also autoxidize [rapidly, from their ketoaminemethylol intermediates by a mechanism analogous to simple monosaccharide autoxidation (Wolff & Dean, 1987*b*)], and this process may also contribute to the fragmentation component which is refractory to inhibition by antioxidants.

Protein fragmentation, tissue damage and glycation

Fragmentation of protein by monosaccharides has been observed previously, but not equated with free-radical damage (Beswick & Harding, 1987). The possibility has, however, been discussed in the context of lens crystallins (Wolff & Dean, 1988) and the protein fragments found in cataract (Wolff *et al.*, 1986), for which age and diabetes are risk factors (Harding & van Heyningen, 1987). In addition, the exposure of lens protein to glucose *in vitro* is known to stimulate protein thiol oxidation (Stevens *et al.*, 1978).

The widespread study of glycation in relation to diabetes and ageing is based on the explicit or implicit assumption that functional and structural alterations resulting from the exposure of macromolecules to hyperglycaemic levels of glucose are caused by the covalent attachment of glucose to protein amino groups, with resultant changes in surface charge, hydrogen bonding capacity, cellular recognition and/or the formation of complex products capable of cross-linking (Cerami, 1986; Pongor *et al.*, 1984; Harding, 1985). The present study shows, in contrast, that the incorporation of monosaccharide into protein can be dissociated from fragmentation and conformational change associated with the exposure of protein to glucose. We have also shown that levels of glycofluorophore formation cannot be equated with extent of protein structural change.



Scheme 1. The contribution of glucose autoxidation (Wolff *et al.*, 1984; Wolff & Dean, 1987*a*,*b*) and hydroxyl radical production to glucose-induced protein damage

The contribution of ketoaldehyde-amine autoxidation (Wolff & Dean, 1987*b*) to site-specific damage is suggested. (With apologies to Roy Lichtenstein's 'Whaam!').

Glucose autoxidation, hydroxyl radical production and protein structural damage

We propose instead that the structural alterations observed are largely dependent upon hydroxyl radicals produced by glucose autoxidation (see Scheme 1). DETAPAC inhibits protein damage by inhibiting the underlying process of glucose autoxidation, and by inhibiting hydroxyl radical production from hydrogen peroxide, generated as a result of glucose oxidation (Wolff *et al.*, 1984; Wolff & Dean, 1987*a*,*b*). In contrast, sorbitol, benzoate and deoxyribose compete with protein for hydroxyl radicals produced by glucose autoxidation as a result of the reaction of transition metal with H_2O_2, but do not inhibit glucose autoxidation *per se*. Cu^{2+} stimulates protein structural change by stimulating hydroxyl radical production from accumulated H_2O_2. Iron salts also stimulate protein fragmentation by glucose (results not shown), but the insolubility of Fe^{3+}, and the experimental complication of the need for EDTA to buffer it in solution, together with the possibly greater relevance of Cu^{2+} to diabetes and ageing, led us to focus on the latter transition metal in these studies.

Glycation studies suggest antioxidant therapy

The present study confirms that free radical and peroxide production have to be considered in any circumstance in which biological structures are exposed to elevated levels of monosaccharide. We conclude that, if experimental glycation studies are an appropriate model for the sequelae of diabetes and ageing, then monosaccharide autoxidation and inappropriate oxidation must play a role in their pathophysiology, consistent with the evidence for increased oxidative stress in these circumstances (Wolff, 1987). This would seem to provide further support for the potential of agents which minimize biological autoxidative processes and their consequences in the therapy of non-malignant diseases associated with diabetes mellitus and ageing (Wolff, 1987).

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